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Chlamydiaceae and *Chlamydia*-like organisms in the koala (*Phascolarctos cinereus*)—Organ distribution and histopathological findings

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Chlamydiaceae and *Chlamydia*-like organisms in the koala (Phascolarctos cinereus)—Organ distribution and histopathological findings

Zusammenfassung

Chlamydien verursachen in Koalas lebensbedrohliche Erkrankungen, die zu Blindheit und Sterilität führen. Über die systemische Verteilung der Chlamydien in den inneren Organen ist nur wenig bekannt und Daten hinsichtlich pathologischer Läsionen sind begrenzt. Das Ziel dieser Studie war es, eine gründliche Untersuchung der inneren Organe bei 23 Koalas durchzuführen und ihre histopathologischen Läsionen mit molekularen Erkennungsmethoden zu vergleichen. Mit PCR wurden zwei Koalas positiv für *Chlamydia pecorum* getestet, während immunhistochemische Marker für *Chlamydiaceae* in 10 Gewebeproben erkannt wurden. Etwas unerwartet waren positive Markierungen im Gastrointestinaltrakt, einschliesslich der Kloake sowie in Lunge und Milz, was auf eine systemische Ausbreitung der Infektion hindeutet. Uncultured *Chlamydiales* wurden in sieben Koalas durch PCR detektiert und vier Tiere davon litten an einer plasmazellulären Enteritis unbekannter Ätiologie. Ob das Auffinden von *Chlamydia*-like Organismen im Gastrointestinaltrakt mit der plasmazellulären Enteritis verbunden ist, bleibt unklar und ist spekulativ. Wie jedoch kürzlich in einem Mausmodell gezeigt werden konnte, könnte der Gastrointestinaltrakt eine Rolle spielen als Ort für persistente Chlamydien-Infektionen und eine Quelle für eine Reinfektion des Genitaltraktes darstellen.

Schlüsselwörter: *Chlamydia pecorum*, Magendarminfektion, Koala, *Phascolarctos cinereus*,

Pathologie, uncultured *Chlamydialesa*

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Summary

Chlamydial infections in koalas can cause life-threatening diseases leading to blindness and sterility. However, little is known about the systemic spread of chlamydiae in the inner organs of the koala, and data concerning related pathological organ lesions are limited. The aim of this study was to perform a thorough investigation of organs from 23 koalas and to correlate their histopathological lesions to molecular chlamydial detection. To reach this goal, 246 formalin-fixed and paraffin embedded organ samples were investigated. By PCR, two koalas were positive for *Chlamydia pecorum* whereas immunohistochemical labelling for *Chlamydiaceae* was detected in 10 tissues. The majority of these (n=6) had positive labelling in the urogenital tract related to histopathological lesions such as cystitis, endometritis, pyelonephritis and prostatitis. Somehow unexpected was the positive labelling in the gastrointestinal tract including the cloaca as well as in lung and spleen indicating systemic spread of infection. Uncultured *Chlamydiales* were detected in seven koalas by PCR, and four of these suffered from plasmacytic enteritis of unknown aetiology. Whether the finding of *Chlamydia*-like organisms in the gastrointestinal tract is linked to plasmacytic enteritis is unclear and remains speculative. However, as recently shown in a mouse model, the gastrointestinal tract might play a role being the site for persistent infections and being a source for reinfection of the genital tract.

Key words: *Chlamydia pecorum*, intestinal infection, koala, *Phascolarctos cinereus*, pathology, uncultured *Chlamydiales*

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Chlamydiaceae and *Chlamydia*-like organisms in the koala (*Phascolarctos cinereus*)—Organ distribution and histopathological findings

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ABSTRACT

Chlamydial infections in koalas can cause life-threatening diseases leading to blindness and sterility. However, little is known about the systemic spread of chlamydiae in the inner organs of the koala, and data concerning related pathological organ lesions are limited. The aim of this study was to perform a thorough investigation of organs from 23 koalas and to correlate their histopathological lesions to molecular chlamydial detection. To reach this goal, 246 formalin-fixed and paraffin embedded organ samples from 23 koalas were investigated by histopathology, *Chlamydiaceae* real-time PCR and immunohistochemistry, ArrayTube Microarray for *Chlamydiaceae* species identification as well as *Chlamydiales* real-time PCR and sequencing. By PCR, two koalas were positive for *Chlamydia pecorum* whereas immunohistochemical labelling for *Chlamydiaceae* was detected in 10 tissues out of nine koalas. The majority of these ($n = 6$) had positive labelling in the urogenital tract related to histopathological lesions such as cystitis, endometritis, pyelonephritis and prostatitis. Somehow unexpected was the positive labelling in the gastrointestinal tract including the cloaca as well as in lung and spleen indicating systemic spread of infection. Uncultured *Chlamydiales* were detected in several organs of seven koalas by PCR, and four of these suffered from plasmacytic enteritis of unknown aetiology. Whether the finding of *Chlamydia*-like organisms in the gastrointestinal tract is linked to plasmacytic enteritis is unclear and remains speculative. However, as recently shown in a mouse model, the gastrointestinal tract might play a role being the site for persistent chlamydial infections and being a source for reinfection of the genital tract.

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1. Introduction

The koala (*Phascolarctos cinereus*) is an Australian arboreal herbivorous marsupial and the only living member of the *Phascolarctidae* family. Nowadays, koala free-range populations are mainly found in coastal areas of the mainland's eastern and southern regions of Australia such as Queensland, New South Wales, Victoria, and South Australia (Polkinghorne et al., 2013). Chlamydiae occur worldwide as obligate intracellular gram-negative bacteria with a biphasic development cycle affecting a wide range of animals, including marsupials, birds and humans. Target cells for chlamydial replication are mucosal epithelial cells of respiratory, gastrointestinal, urogenital tract or conjunctival epithelium as well as trophoblastic epithelium of the placenta and monocytes and macrophages

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(Shewen, 1980; Longbottom and Coulter, 2003; Pospischil et al., 2010). To date, the genus *Chlamydia* contains nine species including *C. pneumoniae* and *C. pecorum*, the two chlamydial species that can infect koalas (Everett et al., 1999; Kuo et al., 2011). *Chlamydia pecorum* is the most prevalent and most virulent chlamydial species in the koala followed by *Chlamydia pneumoniae*. Novel *Chlamydia*-related bacteria also exist in the koala, but these remain uncultured and have not yet been formally classified (Jackson et al., 1999; Devereaux et al., 2003).

Chlamydial infection in koalas can manifest as ocular and/or urogenital tract diseases, but can also cause respiratory infection. Inflammation of the mucosal surface of the eye is characterised by serous discharge, blepharospasm and hyperaemia of the conjunctiva and sclera followed by purulent discharge, conjunctival hyperplasia and fibrosis. The cornea can be affected as well in some chronic cases and shows an opacity caused by oedema, sometimes with pigmentation. In severe cases the globe may rupture and collapse. Inflammation of the urinary tract is called "wet bottom" or "dirty tail" because of the brown urine staining and wetness of the fur in this region caused by cystitis with subsequent incontinence and loss of the bladder function. This might be complicated by a secondary bacterial and/or yeast dermatitis. However, in females, sterility might be the only hint that infection of the reproductive tract has taken place (Cockram and Jackson, 1981; McColl et al., 1984; Brown et al., 1987; Dique et al., 2003; Polkinghorne et al., 2013).

The prevalence of chlamydial infections in koala populations in Queensland, New South Wales and Victoria ranges from 0% (in some isolated island populations that started by translocation of presumably healthy, *Chlamydia*-free animals) up to 100% in other regions (Polkinghorne et al., 2013). The transmission route of *Chlamydia* in koalas is not entirely clear but clinical observations suggest that the transmission is through sexual contact and from mother to joey (Jackson et al., 1999). Most recent studies investigated ocular and genital swabs by PCR methods and/or described the clinical symptoms (Jackson et al., 1999; Devereaux et al., 2003; Markey et al., 2007). In contrast, fewer data is available on the tissue distribution of *Chlamydia* and related histopathological changes in inner organs. In the present study, we performed investigations of the inner organs of 23 koalas using different PCR methods targeting *Chlamydiaceae*, as well as *Chlamydia*-like organisms, and compared the results with histopathological and immunohistochemical findings.

2. Materials and methods

2.1. Sampling

In total, 285 tissue samples embedded in 246 formalin-fixed and paraffin embedded (FFPE) blocks from 23 koalas were investigated in this study. Details of the 23 animals including the type of investigated organs are summarised in Table 1. The first group of koalas (animal nos. 1–10)

Table 1
Details of investigated koalas ($n = 23$) including animal number and identification, sex, origin and number of available organ samples.

Animal		Sex	Origin	UGT[a]	GIT[b]	Eye	Lymphatic organs[c]	Lung	Liver	Heart	Endocrine organ[d]	Brain	Skin	Connective tissue	Musculature
1	B13 003130	F	A	4	3		3	1	1	1	1				
2	B12 023188	F	A	1	5		2		1	1	2		1		
3	B12 030826	F	A	3	6		2	1	2	2	2	2			
4	B11 064511	M	A	2	3		2	1	1	1					
5	B11 027258	F	A	2	4		2	1	1	1	1				
6	B12 047810	M	A	3	5		4	1	1	2	2	2			
7	B12 028638	M	A	6	6		2		1	1					
8	B11 025924	F	A	3	6		4	1	1	1					
9	B11 068025	M	A	3	3		3	1	1			3	2		
10	B11 069090	M	A	2	2		2	1	1	1		4			
11	95/392	F	B	2											
12	95/80	F	B	2	1	1									
13	APO1	M	B	4	4		1	1	1		1				
14	APO2	F	B	2	4		1	1	1	1					
15	APO3	NA	B			1									
16	APO4	NA	B			1									
17	APO5	M	B	4	2		1	1	1	1	1				
18	APO6	M	B	4	3		2	1	1	1					
19	APO7	M	B	4	4		1	1	1	1			1		
20	APO8	F	B	3	5	3	2	1	1	1					
21	APO9	M	B	4	5	2	2	1	1	1					
22	APO10	F	B	3	5	2	2	1	1	1					
23	Koala1	NA	B	1	8		4	1	1		2		2	2	1
Total ($n = 23$)				62	84	10	42	17	20	18	12	11	6	2	1

NA = not available, F = female, M = male.
(A) Endeavour Veterinary Ecology, Toorbul, QLD, Australia.
(B) Moggill Koala Hospital, Brisbane, Australia.
[a] UGT = urogenital tract (kidney, urinary bladder, uterus, testis, prostate).
[b] GIT = gastrointestinal tract (stomach, small and large intestine, caecum, cloaca).
[c] Lymphatic organs: lymph nodes, spleen, thymus.
[d] Endocrine organs: pancreas, adrenal gland.

originated from Endeavour Veterinary Ecology, Toorbul QLD, Australia. These animals were necropsied between April 2011 and January 2013. Of these, eight animals had to be euthanised and two animals were found dead. Archived tissue of animals no. 11 and no. 12 dated back from 1995, but did not contain any further information. The last group of koalas ($n = 11$) (animal nos. 13–23) had an unknown clinical history. Of these, organ samples were collected at the Moggill Koala Hospital, Brisbane, QLD, Australia and these animals were either dead on arrival or were euthanised between February and September 2000.

2.2. DNA extraction

Thirty micrometer sections of formalin-fixed and paraffin-embedded tissue blocks ($n = 246$) were cut and deparaffinised in xylene by centrifugation at 13,800 × *g* for 5 min. The supernatant containing the xylene was removed, followed by two repetitions of adding ethanol, centrifugation at 14,800 × *g* for 5 min and removal of the supernatant. The remaining pellet was lysed with proteinase K (20 mg/ml, Roche Diagnostics, Mannheim, Germany) on a thermomixer at 55 °C and 550 rpm overnight. Using the commercial DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), DNA was extracted according to the manufacturer's instructions. All samples were examined with the Nanodrop® 1000 Version 3.7.1 (Thermo Fisher Scientific, Waltham, USA) to determine DNA quantity and quality.

2.3. Chlamydiaceae real-time PCR

All paraffin block samples ($n = 246$) were tested in duplicate using a 23S rRNA gene-based *Chlamydiaceae* family-specific real-time PCR as previously described (Ehricht et al., 2006) on an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA). Primers Ch23S-F (5′-CTGAAACCAGTAGCTTATAAGCGGT-3′), Ch23S-R (5′-ACCTCGCCGTTTAACTTAACTCC-3′) and probe CH23S-p (FAM-CTCATCATGCAAAAGGCACGCCG-TAMRA) (Microsynth, Balgach, Switzerland) were used to generate a 111-bp product specific for members of the family *Chlamydiaceae* as well as a 177-bp product for the internal amplification control by using primers EGFP-1-F (5′-GACCAACTACCAGCAGAACAC-3′), EGFP-10-R (3′-CTTGTACAGCTCGTCCATGC-5′) and probe EGFP-HEX (HEX-AGCACCCAGTCCGCCCTGAGCA-BH). To achieve a final volume of 25 μl for each sample, 2.5 μl of extracted DNA, 12.5 μl of 2× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) and a final concentration of 5 pmol/μl of each primer and the probe were added. The cycling program started with the initial denaturation (95 °C for 20 s), followed by 45 cycles of denaturation (95 °C for 3 s) and amplification (60 °C for 30 s). The threshold value was calculated automatically. A Ct value <38 was considered as positive, a Ct value >38 as questionable positive. If the mean Ct value was a questionable positive, the *Chlamydiaceae* real-time PCR was repeated. All samples with positive PCR results were further tested by the ArrayTube (AT) Microarray.

2.4. Chlamydiales real-time PCR and sequencing

All samples were investigated by a 16S ribosomal DNA (rDNA)-based *Chlamydiales*-specific real-time PCR (Lienard et al., 2011). All samples with a Ct value <36 were sequenced and sequences were compared with the GenBank database using the BLAST server from the National Centre for Biotechnology (http://www.ncbi.nlm.nih.gov/blast/).

2.5. ArrayTube (AT) microarray for species identification of Chlamydiaceae

Positive samples by real-time PCR for *Chlamydiaceae* ($n = 9$) were further investigated by the species-specific 23S ArrayTube (AT) Microarray (Alere Technologies GmbH, Jena, Germany) as described previously (Borel et al., 2008).

2.6. Histopathology

For histological investigation, two micrometer sections of formalin-fixed and paraffin-embedded tissue (FFPE) of all tissue blocks ($n = 246$) were prepared and stained with haematoxylin and eosin (HE) with the Tissue-Tek® Prisma instrument (Sysmex Digitana AG, Horgen, Switzerland). Histological lesions were assessed microscopically from HE-stained tissue sections and were classified according to their degree and type/age in the investigated organs. The degree of tissue infiltration by inflammatory cells (neutrophils, macrophages, lymphocytes and plasma cells) was assessed semi-quantitatively and was classified as mild, moderate or severe. In mildly inflamed tissues, scattered (up to 20) leucocytes were present in the tissue parenchyma, in the epithelial layer and in the lamina propria or both. In moderate inflammation, more leucocytes were present (20–50), sometimes in aggregates. In severe inflammation, multifocal to confluent (more than 50) accumulations of leucocytes were present affecting the tissue parenchyma or the epithelial layer infiltrating the underlying lamina propria and submucosa. The type/age of lesions was grouped into active, chronic and chronic-active. Active lesions were characterised by neutrophilic infiltration. Chronic inflammatory lesions had infiltration by macrophages, lymphocytes and plasma cells. The chronic-active type was a mixture thereof. Additional changes such as loss of epithelial layer by ulceration and/or necrosis or epithelial proliferation were recorded separately.

2.7. Chlamydiaceae IHC

All FFPE blocks ($n = 246$) were investigated by immunohistochemistry for the presence of chlamydial antigen. A *Chlamydiaceae* family-specific mouse monoclonal antibody (Progen Biotechnik GmbH, Heidelberg, Germany) was used that is directed against the chlamydial lipopolysaccharide and a Detection kit (Dako ChemMate, Dako, Glostrup, Denmark) according to the manufacturer's instruction. Briefly, slides were deparaffinised in xylene and rehydrated through graded ethanol to water followed by a 10-min enzyme digestion using proteinase K (Pronase,

Dako). To block the endogenous peroxidase activity, a peroxidase-blocking solution was applied for 5 min at room temperature. Then the slides were incubated for 30 min with the primary antibody (1:200 in antibody diluent) followed by the link-antibody and the horseradish peroxidase-conjugated streptavidin for 10 min each. Finally, the slides were developed in 2-amino-9-ethylcarbazole substrate solution for 10 min and counterstained with haematoxylin. A negative control was performed for each section by replacing the primary antibody by antibody diluent (Dako). Experimentally infected intestinal tissue of gnotobiotic piglets with porcine *C. suis* stain S45 were used for positive control (Guscetti et al., 2000).

3. Results

Details of the 15 koalas positive for *Chlamydiales* including clinical history, histopathological diagnosis, preliminary tests, immunohistochemistry and PCR results for chlamydiae are shown in Table 2. In summary, two koalas were positive for *C. pecorum* and 11 koalas were positive for *Chlamydiaceae* by IHC and/or real-time PCR. Positive IHC labelling was present in the urogenital tract ($n = 6$), gastrointestinal tract ($n = 2$), spleen and lung in one koala. *C. pecorum* was associated with cystitis and metritis in a female koala (no. 5) and in a male koala (no. 7) with cystitis, prostatitis and gastroenteritis. Positivity for *Chlamydiaceae* by IHC and/or real-time PCR was associated with cystitis ($n = 3$), prostatitis ($n = 2$), enteritis/proctitis ($n = 1$) and splenitis/lymphadenitis ($n = 1$). Uncultured *Chlamydiales* were detected in seven koalas. Positivity of uncultured *Chlamydiales* was associated with enteritis/typhlocolitis ($n = 3$), cystitis ($n = 2$), prostatitis ($n = 2$), metritis ($n = 1$) and glomerulonephritis ($n = 1$). Mixed infections with *Chlamydiaceae* and uncultured *Chlamydiales* were present in five koalas. The remaining eight koalas were negative for *Chlamydiales* by any post-mortem investigations (Table 3).

3.1. Chlamydiaceae real-time PCR and ArrayTube (AT) microarray

Extracted DNA from 246 samples originating from 23 koalas was investigated for *Chlamydiaceae* by real-time PCR. Of these, nine samples out of seven koalas were considered as positive.

These nine samples were investigated by the species-specific 23S Array Tube (AT) Microarray. The presence of *C. pecorum* was confirmed in two out of nine samples from animal No. 7, whereas in the other seven samples no species determination was possible by AT Microarray.

3.2. Chlamydiales real-time PCR and sequencing

All 246 samples were investigated by the 16S ribosomal DNA (rDNA)-based *Chlamydiales*-specific real-time PCR. A total of 19 samples out of eight animals were considered positive (Ct value < 36) and were sent for sequencing. *C. pecorum* was detected in one case (koala no. 5). In koalas no. 1, 3, 4, 7, 11, 14 and 20, uncultured *Chlamydiales* were detected. Koala no. 7 showed sequence similarity with *C. pecorum* and uncultured *Chlamydiales* in the urogenital and gastrointestinal tract whereas all other *Chlamydiales* were belonging to the *Chlamydiales* order, but outside the *Chlamydiaceae* family (Supplementary figure). The PCR product of koala no. 20 (eye sample) resulted in two different sequences as indicated in Table 2.

3.3. Histopathology

Histopathological lesions were detected in 20 of the 23 koalas. The affected organs were the urinary bladder ($n = 9$), the gastrointestinal tract ($n = 6$), the prostate ($n = 5$), the kidney ($n = 6$), the lymph nodes ($n = 4$), the uterus ($n = 3$), the eye ($n = 3$), the spleen ($n = 1$), the lung ($n = 1$), and the liver ($n = 1$). The severity of the cystitis ranged from mild ($n = 3$) to moderate ($n = 6$) and the type was chronic ($n = 5$), chronic-active ($n = 3$) and active ($n = 1$). Chronic cystitis was characterised by infiltration with macrophages, lymphocytes and plasma cells. In active cystitis, a moderate number of neutrophils could be seen. Three female koalas suffered from metritis and/or endometritis (Fig. 1A). The type and severity was moderate and chronic ($n = 1$), moderate and chronic-active ($n = 1$) and mild suppurative ($n = 1$). The chronic endometritis case was characterised by infiltration with macrophages, lymphocytes and plasma cells and loss of the epithelial layer. The mild purulent metritis was characterised by a mild infiltration with neutrophils and the chronic-active type was a mixture thereof. In males, five animals had a prostatitis, of which four cases were active to chronic-active, purulent to mixed cellular and ranged from mild to severe (Fig. 2A). The other case was chronic, plasmacytic and mild. One of the male animals showed a moderate chronic urethritis and peri-urethritis. Cystitis without associated lesions in the female or male genital tract was diagnosed in three koalas (Fig. 3A). Pathological changes in the kidney included pan-glomerular sclerosis ($n = 2$), mild glomerulonephritis ($n = 2$), chronic interstitial nephritis with a severe chronic-active pyelonephritis ($n = 1$, Fig. 4A) and mild chronic-active pyelitis ($n = 1$).

Moderate plasmacytic enteritis was present in six koalas whereas one koala showed a moderate chronic-active proctitis (Fig. 5A). A total of three animals had a (kerato-) conjunctivitis ranging from mild active suppurative conjunctivitis ($n = 1$) to severe chronic or chronic-active, ulcerative or necrotizing and proliferative conjunctivitis. Lymphadenitis was mild and active in one animal and mild and chronic in another three animals. Rare findings were a mild interstitial hepatitis ($n = 1$), a mild interstitial pneumonia ($n = 1$) and a moderate suppurative splenitis ($n = 1$). Non-pathological post-mortem findings included autolysis and congestion (Fig. 6A).

3.4. Chlamydiaceae IHC

Immunohistochemistry was performed in all 246 FFPE tissue blocks. Positive labelling was detected in ten tissues out of nine koalas. The majority of koalas ($n = 6$) were positive in the urogenital tract. Koala no. 5 (Fig. 1B) had single cells positively labelled in the lumen of the uterus, no. 7 (Fig. 2B) showed 2–10 positive cells in the lumen of

Table 2
Results of koalas ($n = 18$) positive for *Chlamydiales* including necropsy findings, previous clinical testing (Clearview test), immunohistochemistry, PCR and sequencing for chlamydiae.

Animal no.	Anamnesis	Diagnosis	Clearview test	IHC	*Chlamydiaceae*-PCR	AT	Pan-*Chlamydiales* PCR	Sequencing
1	Kerato-conjunctivitis, metritis	Metritis, enteritis	Negative	Positive[d]	Negative	ND	Positive[c,d]	Uncultured *Chlamydiales* (98.1% sequence similarity with HM444986)[c], 1F[i] Uncultured *Chlamydiales* (99.3% sequence similarity with JN606074)[d], 1K[i]
3	Episodes of seizures and collapse	Enteritis, typhlo-colitis	NA	Negative	Negative	ND	Positive [c,d,e,f,h]	Sequencing failed[j,c], 3F[i] Uncultured *Chlamydiales* (95.4% sequence similarity with JF660305)[d,h], 3H[i] Uncultured *Chlamydiales* (95.3% sequence similarity with JN701140)[e,f], 3D[i]
4	Unilateral kerato-conjunctivits, mass in epipubic region	Epipubic osteochondroma, enteritis, prostatitis	Positive [a,b]	Negative	Positive[g]	Negative	Positive [c,d,e,f,g,h]	Sequencing failed[j,c,g], 4G[i], 4H[i] Uncultured *Chlamydiales* (100% sequence similarity with JQ860075)[d], 4D1[i] Uncultured *Chlamydiales* (100% sequence similarity with HM444977)[e], 4F[i] Uncultured *Chlamydiales* (99.3% sequence similarity with JQ860075)[f,h], 4B[i]
5	Bilateral kerato-conjunctivitis, cystitis, bilateral reproductive tract disease	Cystitis, metritis	Positive [c]	Positive [c]	Negative	ND	Positive [d]	*Chlamydia pecorum* (100% sequence similarity with D85717)[d], 5F[i]
6	Diabetes	Septicemia	NA	Negative	Positive[d]	Negative	Negative	ND
7	Wet bottom	Cystitis, prostatitis, typhlocolitis, gastritis, enteritis	NA	Positive [c]	Positive[c,d]	*C. pecorum*	Positive[c,d]	Uncultured *Chlamydiales* (89.9% sequencing similarity with JN701140)[c], 7i[i]; *Chlamydia pecorum* (94.7% sequencing similarity with CP004033)[c,d], 7C[i]
8	Poor body condition	Cystitis	Positive[a,b]	Negative	Positive[d,e]	Negative	Negative	ND
10	Found dead	Plasmacytic enteritis	Negative	Negative	Positive[c]	Negative	Negative	ND
11	NA	Cystitis	NA	Positive[c]	Negative	ND	Positive[c]	Uncultured *Chlamydiales* (94.4% sequence similarity with JN701140)[c], K12[i]
12	NA	Cystitis	NA	Positive[c]	Positive[c]	Negative	Negative	ND
13	NA	Cystitis, prostatitis, nephritis and pyelonephritis	NA	Positive[c]	Negative	ND	Negative	ND
14	NA							Glomerulonephritis
NA	Negative	Negative	ND	Positive[c]	Uncultured *Chlamydiales* (94.4% sequence similarity with HQ721208)[c], K06_2[i]			

Table 2 (*Continued*)

Animal no.	Anamnesis	Diagnosis	Clearview test	IHC	*Chlamydiaceae*-PCR	AT	Pan-*Chlamydiales* PCR	Sequencing
17	NA	Prostatitis, panglomerular sclerosis, interstitial pneumonia	NA	Positive[c]	Negative	ND	Negative	ND
20	NA	Interstitial hepatitis, cystitis,						glomerulonephritis, endometritis, lymphadenitis, proctitis
NA	Positive[d]	Positive[f]	Negative	Positive[b]	Uncultured *Chlamydiales* (100% sequence similarity with JX317585)[b], K110_1[i] Uncultured *Chlamydiales* (95.2% sequence similarity with EF693294)[b], K110_2[i]			
22	NA	Splenitis, lymphadenitis	NA	Positive[e,f]	Negative	ND	Negative	ND

ND = not done/NA = not available.
[a] Urine sediment.
[b] Eye.
[c] Urogenital tract.
[d] Gastrointestinal tract.
[e] Lymphatic organs.
[f] Lung.
[g] Liver.
[h] Heart.
[i] Designation of sequences as indicated in the Supplementary Figure 1.
[j] Possibly due to autolysis.

prostate glands, no. 11 had up to ten positive epithelial cells in the genital tract (Fig. 3B), 2–10 positive epithelial cells were seen in the uterus of koala no. 12, and a koala no. 13 displayed 2–10 positive epithelial cells in the epithelium of the renal pelvis (Fig. 4B). Koala no. 17 showed up to ten positive cells in the epithelium in the urinary bladder. In the gastrointestinal tract, 2–10 positive cells were labelled in Koala no. 1 and single positive cells in the epithelium of the cloaca in no. 20 (Fig. 5B). Koala no. 22 (Fig. 6B) showed 2–10 positive cells in the lung (alveolar epithelial cells) and 2–10 positive cells in the spleen (mononuclear cells).

4. Discussion

In the present study, we investigated 246 FFPE blocks containing 285 organ samples from 23 koalas by histopathology, *Chlamydiaceae* immunohistochemistry, *Chlamydiaceae* real-time PCR, ArrayTube (AT) Microarray for species identification of *Chlamydiaceae* as well as by

Table 3
Results of koalas negative for *Chlamydiales* by all post-mortem investigations ($n = 8$).

Animal no.	Anamnesis	Diagnosis	Clearview test
2	Bilateral chronic reproductive tract disease, prolapsed cloaca, chronic low-grade cystitis/nephritis	Acute toxemia/septicemia	Positive[a]
9	Found dead	Enteritis	NA
15	NA	Conjunctivitis	NA
16	NA	Conjunctivitis	NA
18	NA	Panglomerular sclerosis, urethritis and periurethritis, lymphadenitis	NA
19	NA	Pyelitis, prostatitis, cystitis	NA
21	NA	Conjunctivitis, prostatitis, cystitis, lymphadenitis	NA
23	NA	npf	NA

NA = not available.
npf = no pathologic findings.
[a] Urine sediment.

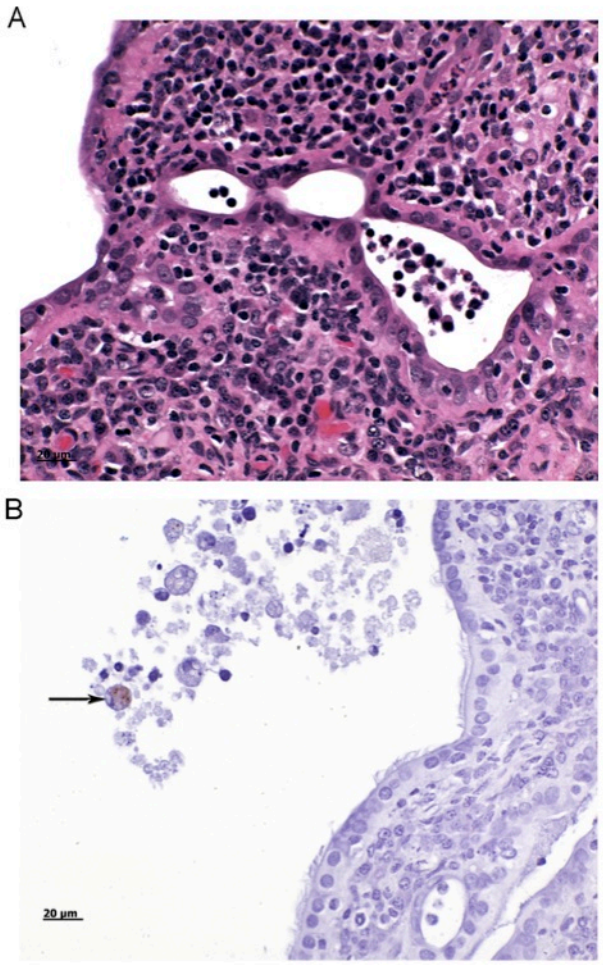

Fig. 1. (A) Uterus, koala no. 5. Histopathology of a case positive for *C. pecorum* by pan-*Chlamydiales* PCR and sequencing (100% homology) in the gastrointestinal tract showing a chronic-active endometritis. The epithelium and subepithelial layer is infiltrated by macrophages, lymphocytes and plasma cells. Lumina of uterine glands contain neutrophils. Haematoxylin and eosin staining. (B) Uterus, koala no 5. Immunohistochemistry, positive labelling of single cells (arrow) in the uterine lumen. AEC/peroxidase method, haematoxylin counterstain.

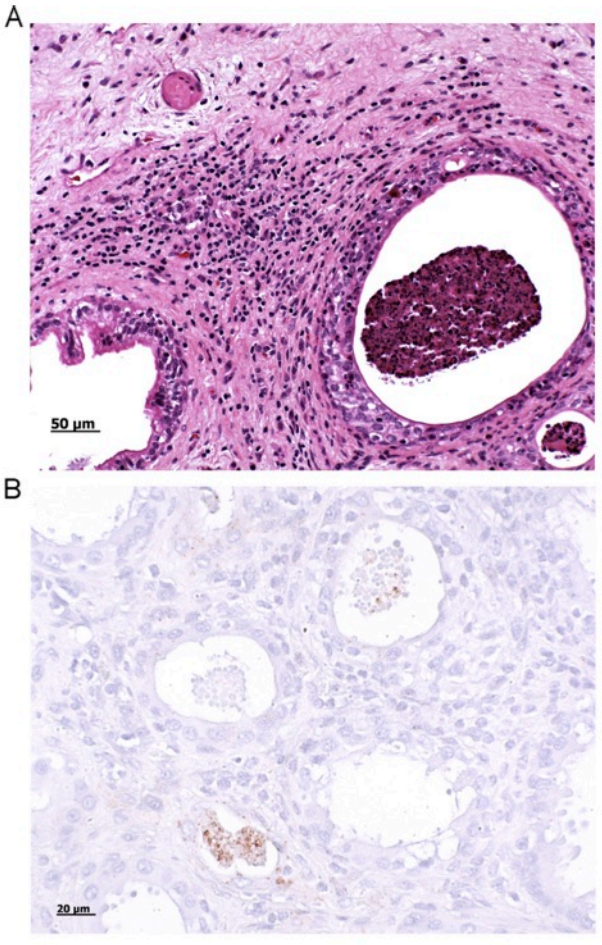

Fig. 2. (A) Prostate, koala no. 7. Histopathology of a case positive for *C. pecorum* by ArrayTube Microarray in the urogenital and gastrointestinal tract showing a chronic-active prostatitis. Lumina of prostate glands contain numerous neutrophils. The periglandular tissue is focally infiltrated by macrophages, lymphocytes, plasma cells and neutrophils. Haematoxylin and eosin staining. (B) Prostate, koala no. 5. Immunohistochemistry, positive granular reaction in the cytoplasm of neutrophils and adjacent epithelium. AEC/peroxidase method, haematoxylin counterstain.

Chlamydiales real-time PCR and sequencing. To the author's knowledge, the chlamydial distribution in the inner organs of koalas infected with either *C. pecorum* and/or *C. pneumoniae* is unknown. Therefore, the present study investigated the distribution of chlamydiae in all inner organs of the 23 animals and compared these results with previous clinical testing (Clearview test) and histopathological lesions. Furthermore, the possible involvement of chlamydiae in the plasmacytic enteritis of koalas was investigated.

In this study, 16 out of 23 koalas had at least one *Chlamydiales*-positive test result, eight of them were positive by two or more tests. Notably, uncultured *Chlamydiales* were more often found than *Chlamydiaceae* and of the latter, two koalas were positive for *C. pecorum* and none for *C. pneumoniae* confirming previous reports showing that *C. pecorum* is more prevalent in koala populations (Jackson et al., 1999). *C. pecorum*-infections in live koalas are mostly determined by investigating conjunctival and/or urogenital swab samples by PCR methods or rapid detection tests (Polkinghorne et al., 2013).

In this study, the Clearview test was performed in six out of 23 koalas prior to euthanasia. The Clearview immunoassay is a qualitative, solid-phase direct antigen detection method with an antibody directed against the *Chlamydiaceae*-family-specific lipopolysaccharide (Hanger et al., 2013). *Chlamydia*-like organisms are likely not detected by the Clearview test as this test is based on the chlamydial lipopolysaccharide (LPS) that is present in *Chlamydiaceae* but little is known about similar LPS-structures in the *Chlamydia*-like organisms. Three out of four Clearview test positive koalas (koala nos. 4, 5 and 8) were confirmed positive by chlamydial PCR and/or immunohistochemistry. Koala no. 4 was positive in the urine sediment as well as in the swab taken from the

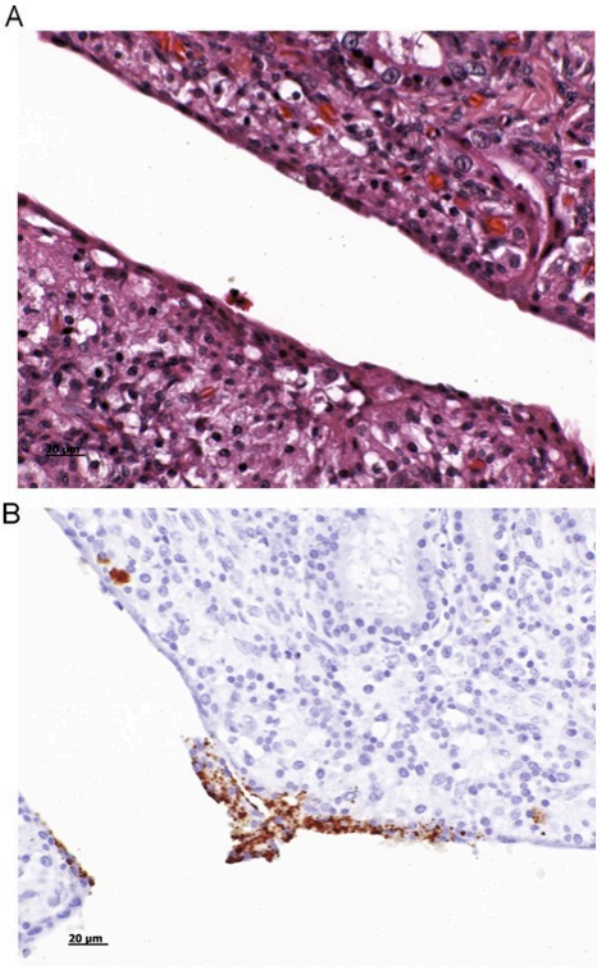

Fig. 3. (A) Genital tract, koala no. 11. Histopathology of a case positive for uncultured *Chlamydiales* by pan-Chlamydiales PCR and sequencing (94.4% similarity to JN701140) in the urogenital tract without pathologic findings in the genital tract. No specific pathological findings such as inflammatory lesions are present. Haematoxylin and eosin staining. (B) Genital tract, koala no 11. Immunohistochemistry. Positive labelling of epithelial cells. AEC/peroxidase method, haematoxylin counterstain.

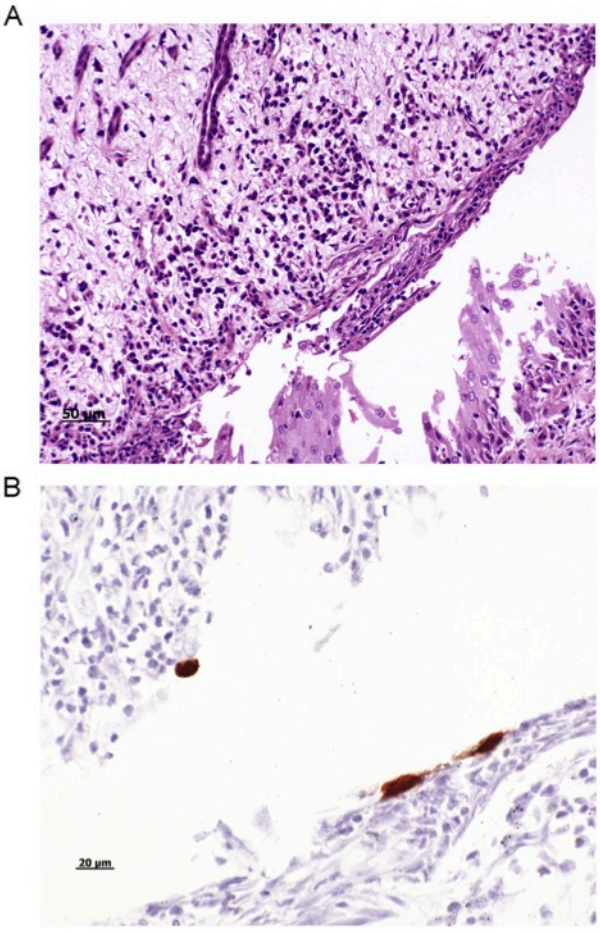

Fig. 4. (A) Kidney, koala no. 13. Histopathology of a case positive for *Chlamydiaceae* immunohistochemistry showing a chronic-active pyelonephritis. The urothelium of the renal pelvis is partially sloughed. The underlying tissue is infiltrated by neutrophils and fewer macrophages and lymphocytes. Haematoxylin and eosin staining. (B) Kidney, koala no. 13. Immunohistochemistry, positive labelling of single cells of the epithelium in the renal pelvis. AEC/peroxidase method, haematoxylin counterstain.

conjunctival sac by the Clearview test, however, PCR results of the inner organs (urogenital tract, gastrointestinal tract, lymphatic organs, lung and heart) revealed only sequences of uncultured *Chlamydiales* and undetermined *Chlamydiaceae* in the liver. Koala no. 5 was positive by the Clearview test in the urogenital tract and this was in accordance with the *Chlamydiaceae*-positive immunohistochemistry in the uterus. Interestingly, this koala was additionally positive by the pan-*Chlamydiales* PCR in the gastrointestinal tract and *C. pecorum* could be confirmed by sequencing. Koala no. 8 was also positive by the Clearview test in the urine sediment and the conjunctival sac and positive in the gastrointestinal tract and lymphatic organs by *Chlamydiaceae* PCR. However, the chlamydial species could not be determined in the latter koala by the AT Microarray possibly due to low DNA copy number or insufficient DNA quality of the FFPE material.

In six animals, positive PCR results corresponded with histopathological lesions of chronic inflammation including lymphocytes, plasma cells and macrophages. The urogenital tract and the gastrointestinal tract were most often affected. The high infection rate of the female and male urogenital tract is well known and reported in the literature (Higgins et al., 2005). In contrast, infections of the gastrointestinal tract, as well as positive labelling in lung and spleen, have not yet been reported.

The present study is the first investigation of all inner organs of *Chlamydia*-infected koalas and showed the systemic spread of *Chlamydiaceae* in nine koalas. *C. pecorum* was found in the urogenital and gastrointestinal tract of koala no. 5 and 7, *Chlamydiaceae* and uncultured *Chlamydiales* were detected in the lung, intestine, spleen and liver by IHC and PCR in 14 koalas. Previous studies focused mainly on the urogenital and gastrointestinal tract of male koalas (Hemsley and Canfield, 2006). Histopathological lesions in *Chlamydia*-infected koalas consisted of inflammation of the

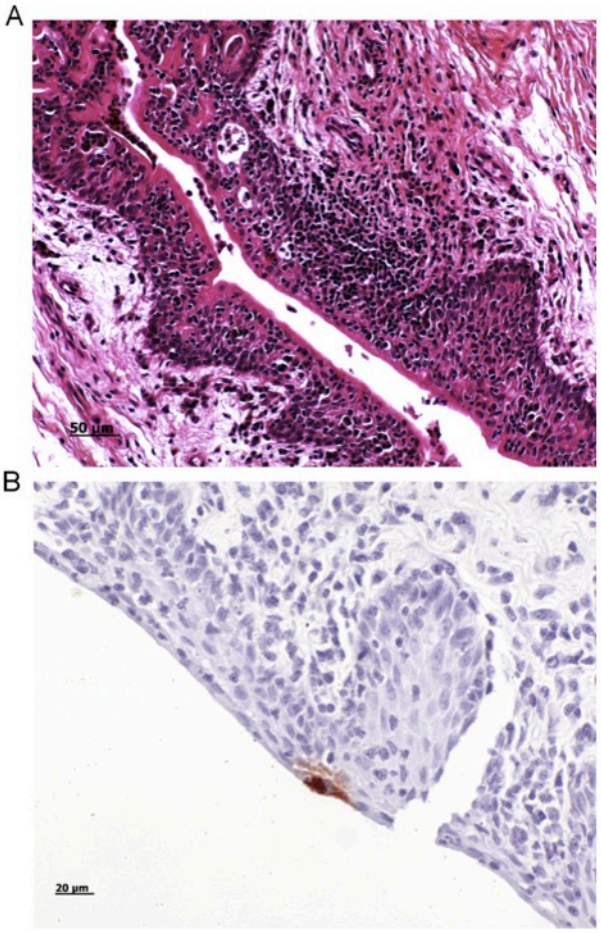

Fig. 5. (A) Cloaca, koala no. 20. Histopathology of a case positive for uncultured *Chlamydiales* by pan-Chlamydiales PCR and sequencing (100% similarity to JX317585) in the eye showing a chronic-active proctitis. The rectal epithelium and subepithelial tissue is diffusely infiltrated by neutrophils, lymphocytes and fewer macrophages. Haematoxylin and eosin staining. (B) Cloaca, koala no. 20. Immunhohistochemistry, positive labelling of single cells in the epithelium. AEC/peroxidase method, haematoxylin counterstain.

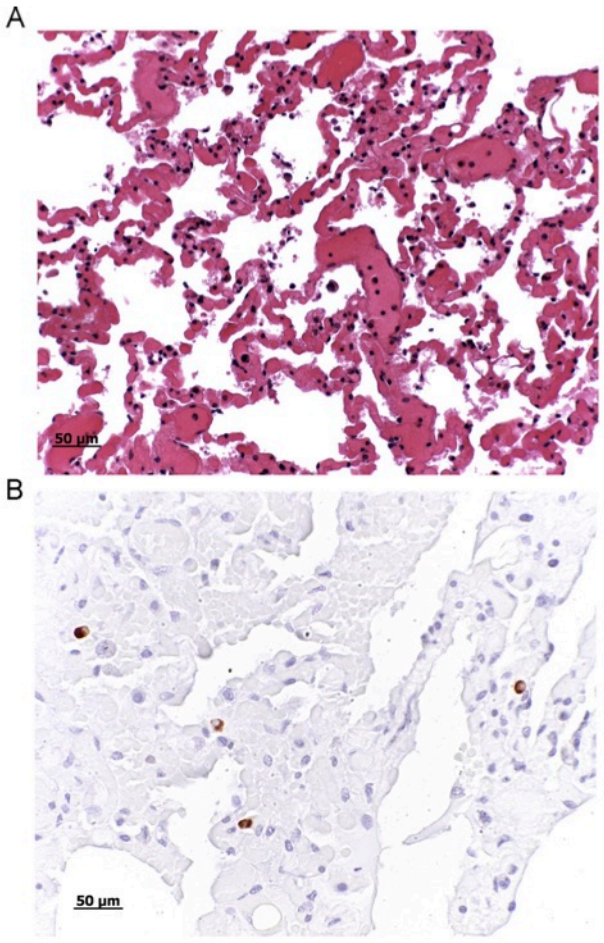

Fig. 6. (A) Lung, koala no. 22. Histopathology of a case positive for *Chlamydiaceae* by immunohistochemistry showing no pathologic findings in the lung. The alveolar septae are of normal architecture, lung capillaries are filled with erythrocytes (congestion). Haematoxylin and eosin staining. (B) Lung, koala no. 22. Immunohistochemistry, positive single cells in the alveolar walls. AEC/peroxidase method, haematoxylin counterstain.

rectal wall, urinary bladder, glandular and urethral prostate and penile urethra but no pathological lesions were diagnosed in the small intestine, colon and caeco-colic junction (Hemsley and Canfield, 2006). Another study investigated the association of uterine and salpingeal fibrosis and chlamydial Heat shock protein (Hsp) 60 and Hsp10 antigen-specific antibodies in *Chlamydia*-infected female koalas (Higgins et al., 2005). However, none of these studies performed a comparative analysis of histology, immunohistochemistry and PCR on the inner organs of koalas. In particular, the present study could show positive labelling for chlamydial antigen in lung and spleen by IHC in a *Chlamydia*-infected koala (no. 22). Notably, this koala had a necropsy report of splenitis and lymphadenitis but remained negative in other organs by PCR. Uncultured *Chlamydiales* were detected in different organs of seven koalas by PCR. A previous study (Devereaux et al., 2003) reported novel chlamydiae in conjunctival and urogenital swabs and tissues by 16S rRNA PCR and sequencing. The same authors also found these new uncultured *Chlamydiales* predominantly as co-infections with *C. pecorum* and/or *C. pneumoniae*. In the present study, only one koala had a mixed infection with *C. pecorum* and uncultured *Chlamydiales* (no. 7) in the urogenital and gastrointestinal tract.

Eight koalas were originally from East Coomera (nos. 1–5, 7, 9–10). Two out of these eight were positive for *C. pecorum* (25%) by PCR and/or IHC. Earlier studies (Polkinghorne et al., 2013) showed prevalence for *C. pecorum* in East Coomera of 33%. However, data from these studies cannot be compared as the study by Polkinghorne et al. (2013) investigated swab samples whereas our study examined archived FFPE material from inner organs. Preliminary clinical testing by the Clearview test in this study was performed on six koalas from the Australian Zoo Wildlife Hospital and of those, four koalas were tested positive.

The relatively low prevalence of *C. pecorum* found in the present study (2 out of 23 koalas positive) was somehow

unexpected and might have different reasons: the affected koalas might have suffered from a chronic/subclinical infection not readily detectable by the investigation of inner organs. The low amount of antigen present in the inner organs detected by IHC (often only single positive cells) might indicate a low level infection. As a consequence, positive labelling might have been lost or PCR was unable to amplify the desired sequence if sections have been taken from different levels of the FFPE blocks. This might also explain the occurrence of non-corresponding results obtained by IHC and PCR, respectively. Another limitation of the study was the age of the archived FFPE blocks. In particular, *C. pecorum* could not be detected in organs of kolas nos. 11–23 by PCR however, these FFPE blocks were more than 10 years old. Preparation and storage of FFPE blocks leads to physical and chemical changes of DNA of the tissue reducing the length of amplifiable PCR fragments (Soldati et al., 2004).

There is no published literature on plasmacytic enteritis in the koala. In the present study, six koalas suffered from plasmacytic enteritis of unknown aetiology. Two of them were negative for chlamydiae (nos. 9 and 10), whereas the other four koalas (nos. 1, 3, 4 and 7) were positive for uncultured *Chlamydiales* by PCR in the gastrointestinal tract and two of these (nos. 1 and 7) were additionally positive for *Chlamydiaceae* (no. 1 by IHC, no. 7 by PCR). Whether the finding of *Chlamydiaceae* and *Chlamydia*-like organisms in the gastrointestinal tract is linked to plasmacytic enteritis is unclear and remains speculative and needs further investigation.

Chlamydiales were detected in the gastrointestinal tract of eight koalas by PCR and/or IHC and of these, *C. pecorum* in two koalas by PCR (no. 5 and 7). Histologically, the gastrointestinal tract was normal in both animals and thus an inapparent intestinal infection with *C. pecorum* might have been present. The gastrointestinal tract is a natural site for chlamydial infections in other animals such as sheep, pigs, cattle, and birds and is suggested as the site for persistent infections (Pospischil et al., 2010). The gastrointestinal tract might also be the source for reinfection of the genital tract as recently shown in a mouse model (Yeruva et al., 2013a). Moreover, antibiotic levels sufficient to treat genital chlamydial infections are ineffective to cure intestinal chlamydial infections as recently published by the same authors (Yeruva et al., 2013b).

5. Conclusion

By the investigation of inner organs from 23 koalas (i) it was confirmed that chlamydial infections are related to cystitis, endometritis, pyelonephritis and prostatitis, (ii) there is evidence of a systemic spread of chlamydial infection, (iii) *Chlamydiales* might be associated with plasmacytic enteritis and (iv) inapparent intestinal infections with *C. pecorum* are prevalent. The gastrointestinal tract might be a reservoir for persistent chlamydial infections in the koala leading to frequent re-infections of the urogenital tract. This finding might also have implications for therapeutic and prophylactic strategies such as vaccine development and antibiotic treatment.

Conflict of interest

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publication of this article.

Full names:*******

Candidatus Parilichlamydia carangidicola clone 25YTK11
Candidatus Similichlamydia latridicola strain 123ST10
Candidatus Amphibiichlamydia ranarum strain AMCS11/3
Candidatus Amphibiichlamydia salamandrae strain AMCS11/2
Candidatus Clavochlamydia salmonicola isolate Br25
Criblamydia sequanensis CRIB-18
Candidatus Syngnamydia venezia
Candidatus Mesochlamydia elodeae strain KV
Candidatus Metachlamydia lacustris strain CHSL
Candidatus Protochlamydia amoebophila UWE25
Protochlamydia naegleriophila strain KNic
Parachlamydia acanthamoebae UV-7
Candidatus Renichlamydia lutjani clone ELO
Rhabdochlamydia crassificans strain CRIB01
Candidatus Rhabdochlamydia porcellionis
Candidatus Fritschea eriococci strain Elm
Simkania negevensis Z
Waddlia chondrophila WSU 86-1044
Candidatus Piscichlamydia salmonis clone C093-1
Chlamydia trachomatis D/UW-3/CX
Chlamydia psittaci 6BC
Chlamydia pneumoniae LPCoLN
Chlamydia pneumoniae AR39
Chlamydia muridarum Nigg
Chlamydia ibidis 10-1398/6
Chlamydia felis Fe/C-56
Chlamydia caviae GPICe
Chlamydia abortus strain S26/3
Chlamydia pecorum PV3056/3
Chlamydia pecorum strain Koala type II
Chlamydia pecorum PV3056/3

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.04.022.

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